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Tannic acid inhibits insulin-stimulated lipogenesis in rat adipose tissue and insulin receptor function in vitro

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Abstract. Tannins occur naturally in relatively abundant amounts in fruits, herbal medicines and common beverages. Thus an understanding of how these polyphenols affect peptide hormone action is of importance. We report here that tannic acid (a hydrolysable tannin) inhibits insulin-stimulated lipogenesis in rat adipose tissue in vitro, with an IC_{50} estimated to be about 350 μM. However, its monomer, gallic acid, did not show a similar inhibitory effect at concentrations up to 1 mM. The inhibition by tannic acid was less evident with higher concentrations of bovine serum albumin in the incubation buffer. This was attributed to the formation of a tannin-protein complex between bovine serum albumin and tannic acid. In a binding assay, it was observed that the specific binding of insulin to its receptor was not inhibited by tannic acid in the concentration range 0–200 μM. However, insulin-stimulated autophosphorylation of the insulin receptor, and receptor-associated tyrosine kinase phosphorylation of RR-SRC peptide, were inhibited by tannic acid at concentrations as low as 25 μM. Our data do not support the current speculation that tannins affect the activity of peptide hormones by binding to them. Therefore, our finding opens up a new perspective in the understanding of the mode of action of tannins on such hormones.

Key words. Tannic acid; autophosphorylation; insulin; tyrosine kinase; lipogenesis.

Insulin plays an important role in homeostasis by regulating the activity or amount of some critical proteins[1]. Its modulation of biological responses is initiated through its binding to the α-subunit of the plasma membrane-bound insulin receptor[2]. The binding stimulates autophosphorylation at specific tyrosine residues in the receptor's β-subunit[3,4], which in turn enhances the tyrosine kinase activity towards exogenous substrates[5–7]. Apart from insulin receptor substrate-1 and the β-subunit of the receptor itself, other physiologically important substrates may include the Shc and p62 proteins from the Ras pathway[8–10].

Insulin is also an effective inhibitor of lipolysis and its action has been shown to correlate with a reduction in protein kinase A activity which closely reflects a decrease in cellular cyclic AMP[11]. Some drugs (sulfonylureas) that are used in the treatment of non-insulin-dependent diabetes can affect cyclic AMP levels, thereby causing direct antilipolysis in adipose tissue[12]. A knowledge of the mechanism of action of drugs would allow a better understanding of tolerance to them, and provide a basis for application.

The tannins are naturally-occurring plant polyphenols and are commonly found in herbal medicines[13,14]. Tannic acid (fig. 1) is a gallotannin comprising 8–10 molecules of gallic acid per molecule of glucose[15]. This hydrolysable tannin occurs naturally in the bark and fruits of many plants and is commonly consumed in beverages such as tea, wine and coffee[16,17]. Our laboratory reported that tannic acid was a potent inhibitor of the hyaluronidase enzyme[18], displayed hypocholesterolemic action in genetically hypercholesterolemic

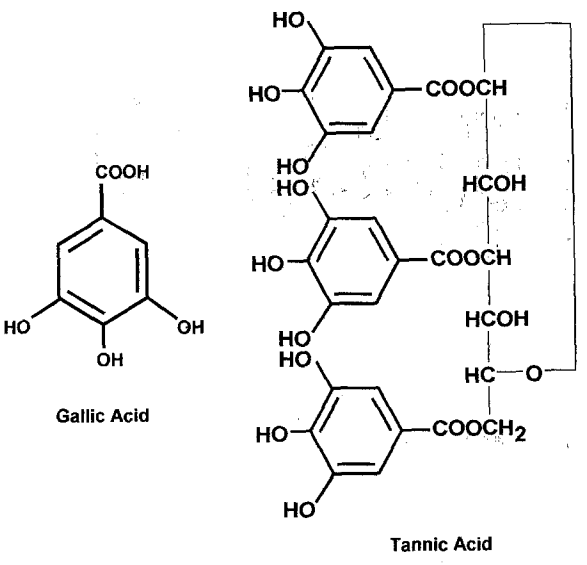

Figure 1. Structures of tannic acid and gallic acid.

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(RICO) rats[19] and caused some favourable changes in the serum lipid parameters of spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats[20]. Some condensed tannins have been shown to inhibit insulin-induced lipogenesis in rat adipocytes while others have been shown to have an enhancing effect. However, the hydrolysable tannins were reported not to have any inhibitory effect on insulin-induced lipogenesis[21].

In view of the fact that no studies have been reported to elucidate the way in which tannins influence insulin action, we present here a study on the effect of tannic acid (a hydrolysable tannin) on insulin-induced lipogenesis in rat adipose tissue, and its mode of action. This report aims to shed some light on the mechanism by which this ubiquitously occurring plant polyphenol exerts its effects on insulin action. Interestingly, tannic acid exerted an inhibitory effect on insulin-induced lipogenesis in rat adipose tissue. This inhibitory effect was, however, not exhibited by its monomer, gallic acid. Tannic acid did not affect insulin action via its binding to the peptide hormone but inhibited both receptor autophosphorylation and receptor-associated tyrosine kinase activity.

Materials and methods

Materials. Fed male Wistar rats (180–220 g) were obtained from the Animal Lab. Centre, National University of Singapore. D-[U-^{14}C]glucose [300 mCi/mmol], [A_{14}-^{125}I]-monoiodoinsulin [$\sim$2000 Ci/mmol], [γ-^{32}P]-adenosine triphosphate [$>$5000 Ci/mmol], monoclonal anti-phosphotyrosine antibody and biodegradable liquid scintillant were purchased from Amersham (Amersham, UK). Recombinant human insulin was obtained from Lilly France SA (Fegersheim, France). Protein Kinase Assay System was purchased from GIBCO BRL (Gaithersburg, MD, USA). Tannic acid was obtained from Extrasynthase (Ganey, France). Bovine serum albumin (BSA) fraction V, collagenase type II (290 units/mg), aprotinin, N-acetyl-D-glucosamine, phenylmethylsulfonyl fluoride (PMSF), adenosine 5′ triphosphate (ATP), sodium dodecyl sulfate (SDS), 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), wheat germ agglutinin-agarose (WGA-agarose) and anti-mouse IgG were bought from Sigma Chemical Co. (St Louis, MO, USA). Silicone oil was from General Scientific (Singapore). Krebs-Henseleit Buffer (KHB), pH 7.4, consisted of 120 mM NaCl, 1.2 mM KH_2PO_4, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ and 2.5 mM $CaCl_2$. All other reagents and chemicals used in this study were of analytical grade.

Lipogenesis assay. Our preliminary work indicated that basal lipogenesis in isolated rat adipocytes was higher in the absence of BSA in the incubation mixture (data not shown). This was not observed for adipose tissue. Since tannic acid readily forms protein-tannin complexes with proteins, which could result in the depletion of BSA in the incubation mixture, lipogenesis was studied in rat adipose tissue instead of isolated adipocytes. Lipogenesis was measured by incorporation of [U-^{14}C]glucose into lipids in rat adipose tissue. Epididymal fat pads from male Wistar rats (180–200 g) were excised and washed free of excess blood and lipids in KHB supplemented with 0.51 mM glucose. The washed adipose tissue was then cut into smaller tissue fragments and divided into portions of about 1 g. Each portion was incubated in KHB supplemented with 0.5% BSA, [U-^{14}C]glucose (4 µCi, 0.51 mM) and varying concentrations of tannic acid, and in the presence or absence of insulin (5 mU/ml) for 2 h at 37 °C, 5% CO_2. Lipid was extracted from the tissue using a chloroform:methanol solvent mixture (2:1, v/v) as described by Folch et al.[22]. The chloroform layer was removed and evaporated to dryness under vacuum. The dried residue (lipid) was weighed, resuspended in biodegradable liquid scintillant and the amount of radioactivity was measured in a Beckman LS 6000LL scintillation counter. The change in glucose concentration at the end of the incubation period was less than 5% under the various conditions studied. Basal incorporation of [U-^{14}C]glucose into lipids was linear for the first 2.5 h.

Preparation of isolated adipocytes. Adipocytes were prepared from epididymal fat pads of male Wistar rats by collagenase digestion (2 mg/ml) in KHB supplemented with 3% BSA and 0.51 mM glucose[23]. Packed cell volume of the concentrated adipocyte stock was estimated prior to cell dilution. This was done by pipetting 100 µl aliquots into separate microfuge tubes, layering with silicone oil ($\sim$50 µl) and centrifuging at 8,500 $\times$ g for 2 min[24]. The volume of the aqueous phase was measured and the packed cell volume inferred from it. The isolated adipocytes were diluted with KHB supplemented with 0.5% BSA and 5.1 mM glucose to give a final packed cell volume of 5%. Cell viability was determined by incorporation of [U-^{14}C]glucose into lipids over a period of 1 h as described by Moody et al.[25]. The incorporation was linear within the first hour after digestion.

Insulin binding assay. This assay was performed using isolated rat adipocytes as described by Gliemann and Sonne[26] with some modifications. Briefly, 400 µl aliquots of cell suspension (packed cell volume = 0.05) were incubated with various concentrations of tannic acid and 50 pM [A_{14}-^{125}I]-monoiodoinsulin in the presence or absence of 10 µM unlabelled insulin (final volume = 500 µl) for 10 min at 37 °C. The reactions were terminated by adding 10 ml of cold saline and 2 ml of chilled silicone oil followed by centrifugation at 2,000 rpm for 2 min. The adipocyte islets layered on top of the silicone oil were sucked into disposable pipette tips and both cell bound and free radioactivity were

counted. Total binding was indicated by the bound/free values obtained for incubations in the absence of excess unlabelled insulin, and the non-specific binding was indicated by those values obtained for incubations in the presence of excess unlabelled insulin. Specific binding was obtained from the difference between the two values (i.e. total minus non-specific).

Purification of insulin receptors. WGA-purified insulin receptors were prepared from rat liver membrane by modifying the procedures described by Meyerovitch et al.[27] and Yarden and Schlessinger[28]. Briefly, rat liver was homogenized and solubilized in 50 mM HEPES (pH 7.4), 1% Triton X-100 supplemented with 2 mM PMSF and aprotinin (10 µg/ml). The suspension was centrifuged at 100,000 × *g* for 60 min and the supernatant was applied to WGA-agarose. The WGA-agarose was pelleted by centrifugation and washed extensively with 50 mM HEPES, 0.1% Triton X-100. The receptors were eluted by suspending in 2 volumes of 50 mM HEPES (pH 7.4), 0.1% Triton X-100 supplemented with 0.3 M N-acetyl-D-glucosamine followed by centrifugation and stored at −80 °C.

Phosphorylation of RR-SRC substrate. WGA-purified insulin receptors were pre-incubated in 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 2 mM $MnCl_2$, 10 mM $MgCl_2$, 100 µM ATP and in the presence or absence of insulin (0.01 U/ml) for 30 min at 22 °C to allow autophosphorylation. This was followed by pre-incubation with tannic acid for another 20 min. Phosphorylation of RR-SRC peptide was assayed using the Protein Tyrosine Kinase Assay System and [γ-^{32}P]ATP (1 µCi).

In vitro autophosphorylation of insulin receptors. Autophosphorylation of WGA-purified insulin receptors was carried out as described by Shisheva and Shechter[29] and the receptors immunoprecipitated by modifying the procedure described by Carrascosa et al.[30]. Insulin receptors (~50 µg total protein) were pre-incubated in 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 2 mM $MnCl_2$, 10 mM $MgCl_2$ and in the presence or absence of insulin (10 mU/ml) at 22 °C for 50 min followed by pre-incubation with tannic acid for another 15 min. Autophosphorylation was initiated by adding 25 µM [γ-^{32}P]ATP (5 µCi). Autophosphorylation was carried out for 10 min and terminated by adding 3 volumes of 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 100 mM NaF, 10 mM $Na_4P_2O_7$, 5 mM EDTA and 2 mM $NaVO_3$ supplemented with 6% BSA. Phosphorylated insulin receptors were incubated overnight with anti-phosphotyrosine antibody (5 µg) at 4 °C. Receptor-antibody complexes were immunoprecipitated with secondary antibody for 2 h at 4 °C. The pellet was resolubilized in electrophoresis treatment buffer, resolved by 7.5% SDS-polyacrylamide gel electrophoresis[31] and identified by autoradiography.

Protein concentrations were estimated using the method of Bradford[32]. All assays were performed either in duplicate or triplicate and all data are presented as

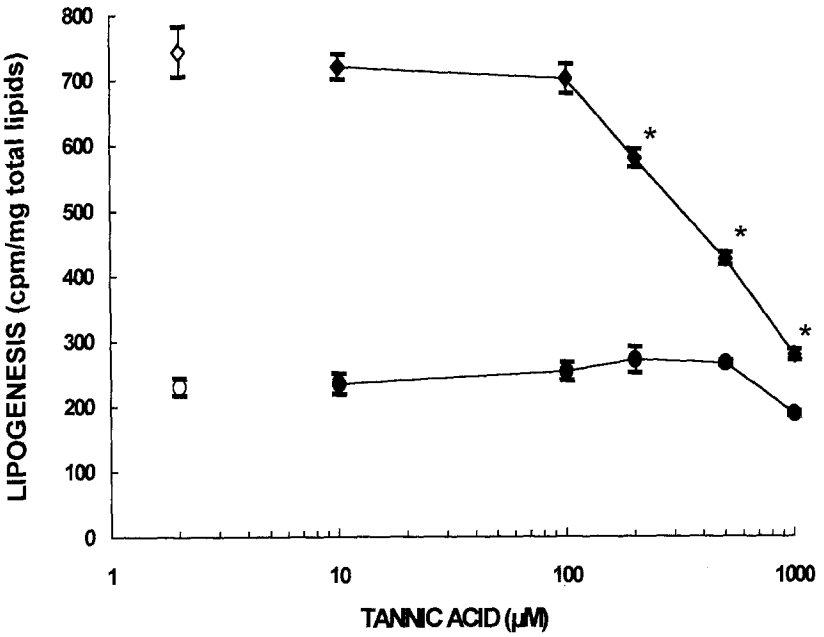

Figure 2. Effect of tannic acid on insulin-stimulated lipogenesis in rat adipose tissue. Rat adipose tissue (~1 g) was incubated in KHB supplemented with 0.5% BSA, [U-^{14}C]glucose (0.51 mM, 4 µCi) and the indicated concentrations of tannic acid in the presence (◆) or absence (●) of insulin (5 mU/ml) for 2 h at 37 °C, 5% CO_2. Total lipid was extracted from the tissue using chloroform:methanol solvent mixture (2:1, v/v) as described by Folch et al.[22]. The chloroform layer was evaporated to dryness, and weighed, and the amount of radioactivity in the total lipid determined. Control samples were incubated either in the presence (◇) or absence (○) of insulin (5 mU/ml) but without tannic acid. Values are means ± SE of 4–5 experiments each performed in duplicate. *$p < 0.01$ (Student's *t*-test) for the difference between samples incubated with tannic acid and controls.

mean ± standard error ($n \geq 3$). Data were analysed using Student's t-test for significance.

Results

Effect of tannic acid and gallic acid on insulin-stimulated lipogenesis. Incubation of rat adipose tissue fragments with increasing concentrations of tannic acid resulted in inhibition of insulin-induced lipogenesis (fig. 2). The inhibition proceeded in a dose-dependent fashion and was evident at concentrations above 100 µM. The IC_{50} was estimated to be about 350 µM. The basal level of lipogenesis was not drastically affected for the range of tannic acid concentrations studied. The inhibition of insulin-induced lipogenesis by tannic acid was found to be dependent on the concentration of BSA present in the incubation buffer (fig. 3). The inhibitory effect of tannic acid was reduced by increasing concentrations of BSA. The activity of adipose tissues incubated in the absence of tannic acid but in the presence of insulin did not differ significantly, whether or not the incubation buffer was supplemented with 0.5% BSA. The tissue fragments did, however, exhibit a significantly higher level of lipogenesis than those incubated with the corresponding BSA supplement in the presence of tannic acid.

To investigate the significance of the gallic acid moiety of tannic acid for insulin-stimulated lipogenesis, rat adipose tissue fragments were incubated with increasing concentrations of gallic acid in the presence of insulin. Gallic acid, unlike tannic acid, did not exhibit any inhibitory effect on insulin-stimulated lipogenesis for concentrations up to 1 mM (data not shown).

Effect of tannic acid on the binding of insulin to the insulin receptor. The specific binding of [A_{14}-^{125}I]-monoiodoinsulin to insulin receptors in isolated adipocytes was not inhibited by tannic acid in the range of concentrations studied (fig. 4). The specific binding was not significantly different for tannic acid concentrations less than 100 µM. However, it was increased for concentrations above 100 µM, suggesting that the specific binding may be dosage-dependent. The tannic acid concentration was limited to 200 µM in this study as recovery of cells was incomplete at higher concentrations. This was possibly due to a reduction in the stability of the isolated adipocytes as a result of BSA being depleted in the incubation buffer by tannic acid.

Effect of tannic acid on the in vitro phosphorylation of RR-SRC substrate by insulin receptor tyrosine kinase. The RR-SRC substrate is a synthetic peptide substrate derived from the phosphorylation site in pp60^{src} and is specific for tyrosine kinases[33]. This substrate has

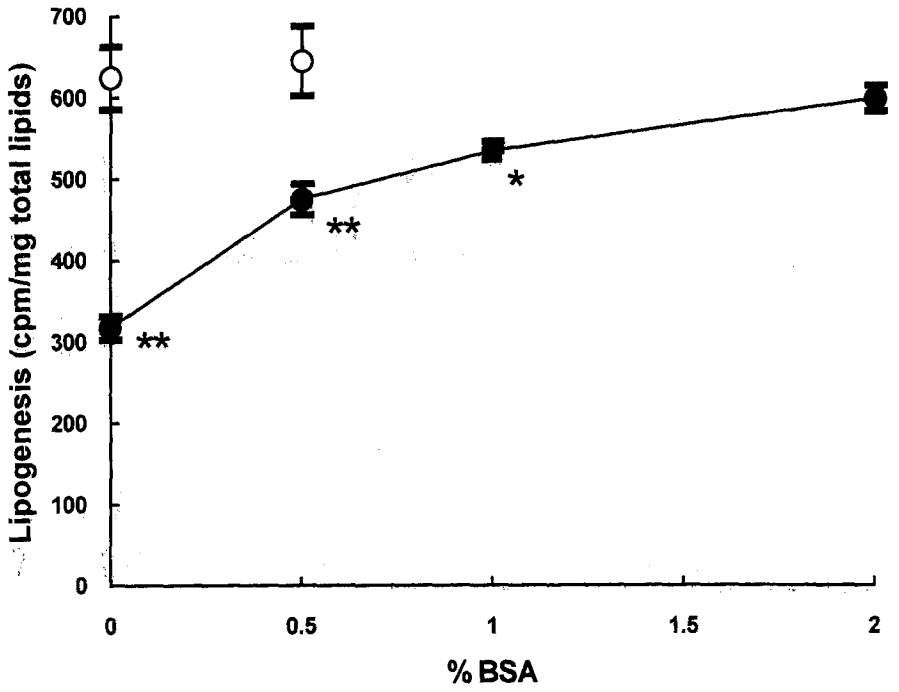

Figure 3. Effect of BSA on the inhibition of insulin-stimulated lipogenesis by tannic acid. Rat adipose tissue (~1 g) was incubated in KHB supplemented with the indicated concentrations of BSA, [U-^{14}C]glucose (0.51 mM, 4 µCi) and insulin (5 mU/ml) in the presence of 350 M tannic acid (●) for 2 h at 37 °C, 5% CO_2. Total lipid was extracted from the tissue using chloroform:methanol solvent mixture (2:1, v/v) as described by Folch et al.[22]. The chloroform layer was evaporated to dryness, and weighed, and the amount of radioactivity in the total lipid determined. Samples were also incubated in the absence of tannic acid (○). Values are mean ± SE of 4 experiments each performed in duplicate. *$p < 0.05$, **$p < 0.01$ (Student's t-test) for the difference between the samples incubated with tannic acid and the control samples incubated in 0.5% BSA but in the absence of tannic acid.

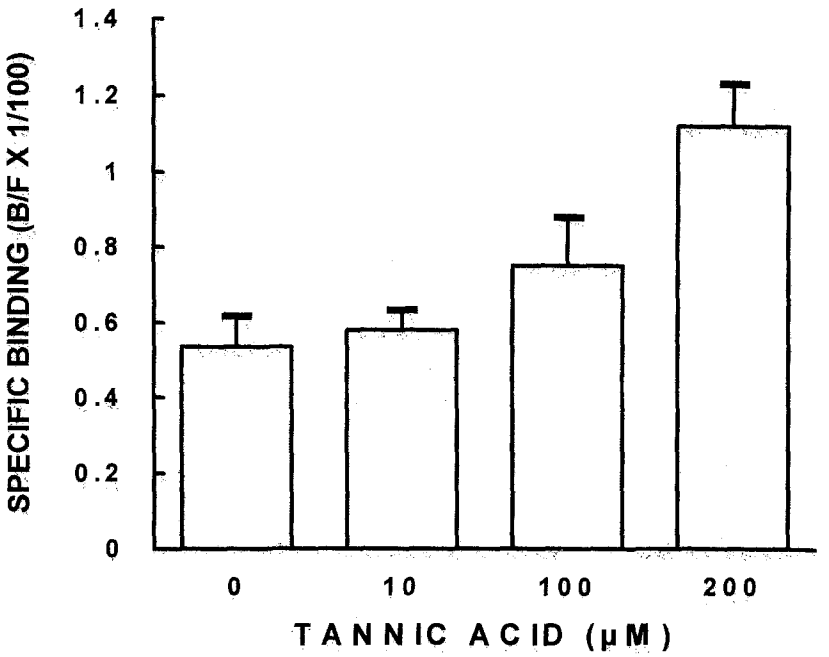

Figure 4. Effect of tannic acid on insulin's binding to the insulin receptor in rat adipocytes. Rat adipocytes (packed cell volume = 0.05) were incubated with the indicated concentrations of tannic acid and 50 pM [A_{14}-^{125}I]-monoiodoinsulin in the presence or absence of 10 μM unlabelled insulin for 10 min at 37 °C. The binding assay was terminated by adding 10 ml cold saline and 2 ml of chilled silicone oil followed by centrifugation at 2,000 rpm for 2 min. The adipocyte islets layering the top of the silicone oil were removed and both cell-bound and free radioactivity were counted. Values are mean ± SE of 4 experiments each performed in quadruplicate.

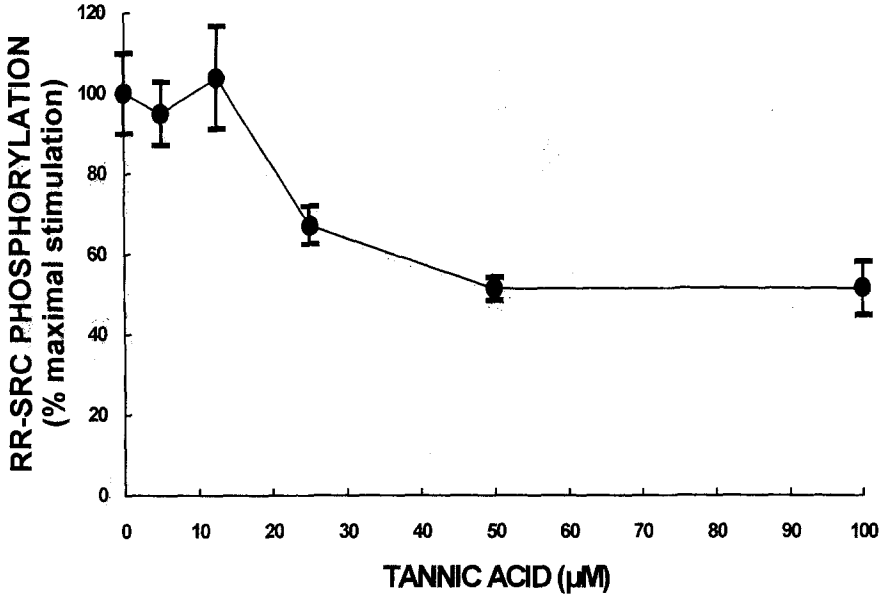

Figure 5. Effect of tannic acid on the phosphorylation of RR-SRC substrate by insulin receptor kinase. WGA-purified insulin receptors from rat liver (~1 μg total protein) were pre-incubated in 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 2 mM $MnCl_2$, 10 mM $MgCl_2$ and 100 μM ATP in the presence or absence of insulin (10 mU/ml) for 30 min at 22 °C followed by pre-incubation with tannic acid for another 20 min. Phosphorylation of RR-SRC peptide was assayed using the Protein Tyrosine Kinase Assay System and [γ-^{32}P]ATP. Values are mean ± SE of 3 experiments each performed in duplicate.

been previously used for measuring insulin receptor tyrosine kinase activity[34]. Tannic acid inhibited the phosphorylation of RR-SRC substrate by insulin receptor tyrosine kinase for concentrations of tannic acid above 12.5 μM (fig. 5). The inhibition of the insulin receptor kinase activity was partial with a maximal inhibition of approximately 50%. This inhibition was not prevented by supplementing the incubation mixture with additional Mg^{2+} and Mn^{2+}, but instead was further increased (fig. 6). Possible interference in the detection of phosphorylated RR-SRC substrate by tannic acid was investigated by adding tannic acid to the

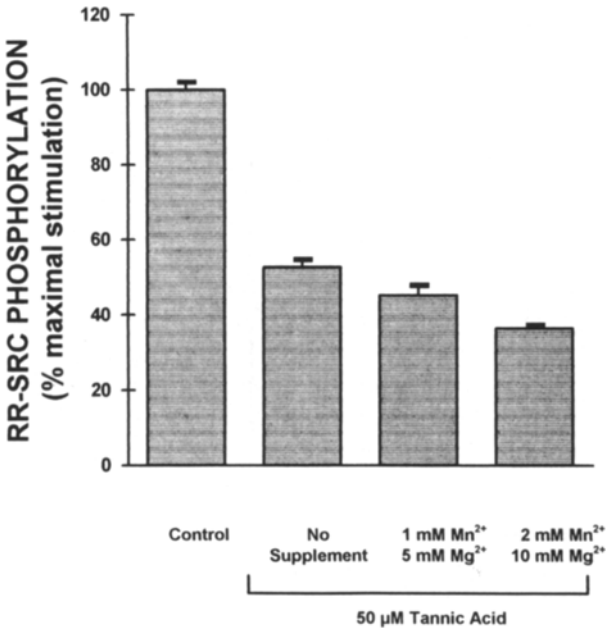

Figure 6. Effect of Mg^{2+} and Mn^{2+} on the inhibition of insulin receptor tyrosine kinase activity by tannic acid. WGA-purified insulin receptors from rat liver (~1 µg total protein) were pre-incubated in 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 2 mM $MnCl_2$, 10 mM $MgCl_2$ and 100 µM ATP in the presence or absence of insulin (10 mU/ml) for 30 min at 22 °C followed by pre-incubation with tannic acid for another 20 min. Phosphorylation of RR-SRC peptide was assayed using the Protein Tyrosine Kinase Assay System with Mg^{2+} and Mn^{2+} supplement and [γ-^{32}P]ATP. Control samples were incubated in the absence of tannic acid and Mg^{2+} and Mn^{2+} supplement. Values are mean ± SE of 3 experiments each performed in duplicate.

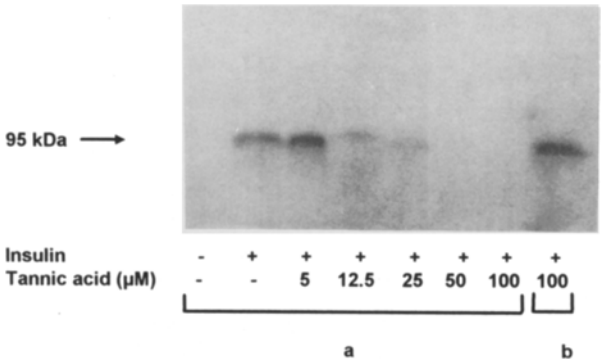

Figure 7. Effect of tannic acid on the autophosphorylation of insulin receptors.
a WGA-purified insulin receptors (~50 µg total protein) were pre-incubated in 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 2 mM $MnCl_2$ and 10 mM $MgCl_2$ with insulin (10 mU/ml) followed by pre-incubation with the indicated concentrations of tannic acid for another 15 min. Autophosphorylation was initiated by adding 25 µM [γ-^{32}P]ATP (5 µCi) and terminated after 10 min by adding 3 volumes of 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 100 mM NaF, 10 mM $Na_4P_2O_7$, 5 mM EDTA and 2 mM $NaVO_3$ supplemented with 6% BSA. Phosphorylated insulin receptors were immunoprecipitated with anti-phosphotyrosine antibodies, resolved by 7.5% SDS-polyacrylamide gel electrophoresis and identified by autoradiography. Control samples were pre-incubated with or without insulin in the absence of tannic acid.
b Insulin receptors were allowed to autophosphorylate in the absence of tannic acid as mentioned above. 100 µM tannic acid was added after termination of autophosphorylation but before immunoprecipitation. Immunoprecipitated insulin receptors were resolved by SDS-polyacrylamide gel electrophoresis and autoradiographed.

reaction mixture just before terminating the assay by TCA addition. Interference in the detection by tannic acid was not observed (data not shown).

Effect of tannic acid on insulin receptor autophosphorylation. WGA-purified insulin receptors were used for studying the effects of tannic acid on insulin receptor autophosphorylation. Tannic acid inhibited autophosphorylation at concentrations above 5 µM (fig. 7a). Autophosphorylation was completely inhibited at tannic acid concentrations above 25 µM. Tannic acid's potential for interfering with the antibody-receptor binding was assessed by adding tannic acid to the incubation mixture after the reaction had been terminated, but before the addition of anti-phosphotyrosine antibody. No apparent interference caused by tannic acid was observed in the immunoprecipitation of phosphorylated insulin receptors (fig. 7b). The inhibition of autophosphorylation as a result of the chelation of divalent cations by tannic acid was excluded by incubating increasing amounts of insulin receptor with the same concentration of tannic acid (fig. 8). The 95 kDa band intensified with increasing amounts of insulin receptor.

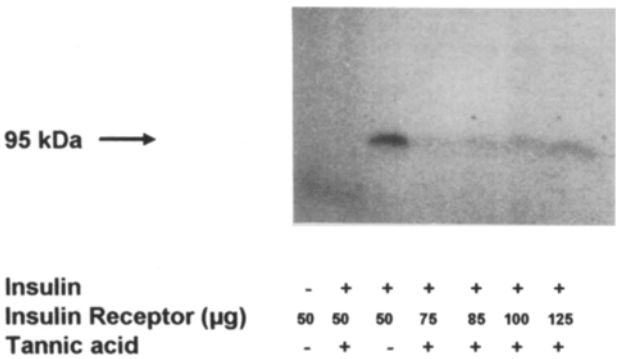

Figure 8. Autophosphorylation of various amounts of insulin receptor in the presence of tannic acid. The indicated amounts of WGA-purified insulin receptors were pre-incubated in 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 2 mM $MnCl_2$ and 10 mM $MgCl_2$ with insulin (10 mU/ml) followed by pre-incubation with 50 µM tannic acid for another 15 min. Autophosphorylation was initiated by adding 25 µM [γ-^{32}P]ATP (5 µCi) and terminated after 10 min by adding 3 volumes of 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 100 mM NaF, 10 mM $Na_4P_2O_7$, 5 mM EDTA and 2 mM $NaVO_3$ supplemented with 6% BSA. Phosphorylated insulin receptors were immunoprecipitated with anti-phosphotyrosine antibodies (10 µg), resolved by 7.5% SDS-polyacrylamide gel electrophoresis and identified by autoradiography. Control samples were pre-incubated with or without insulin in the absence of tannic acid.

Discussion

The understanding of the effects and the mode of action of tannins on peptide hormones is of great nutritional importance, especially as tannins occur naturally in relatively abundant amounts in fruits, herbal medicine, and common beverages such as tea, coffee, and wine[13,14]. Kimura et al.[21] postulated that the inhibitory effect of tannins on insulin-stimulated lipogenesis in rat adipocytes could be attributed to the binding of the tannins to insulin which could then modify the peptide hormone action. In this study, we are able to provide experimental evidence that the inhibitory effects of tannic acid (a hydrolysable tannin) on insulin action did not occur through the binding of tannic acid to insulin. Instead, tannic acid acted on the insulin receptor, resulting in the inhibition of insulin-stimulated autophosphorylation as well as the tyrosine kinase activity associated with the receptor.

Tannic acid was found to inhibit insulin-induced lipogenesis in rat adipose tissue (fig. 2). The IC_{50} was estimated to be about 350 μM. Basal lipogenesis was not drastically affected for the range of tannic acid concentrations studied, thus ruling out the involvement of glucose transporters and possible toxic effects of tannic acid. However, the inhibitory effect of tannic acid was masked when the concentration of BSA in the incubation buffer was increased (fig. 3). BSA is known to bind to tannins readily[35] and has often been used to quantitate tannin[36,37]. In the study by Kumura et al.[21], the rat adipocytes were incubated with hydrolysable tannins in the presence of 5% albumin. The possibility arises that the high concentration of albumin used could have masked potential inhibitory effects of the hydrolysable tannins studied. Further studies of those hydrolysable tannins at low BSA concentrations would be necessary before any conclusion can be drawn about the effects these polyphenols have on insulin-induced lipogenesis. Particular consideration should be given to BSA concentration in studies involving tannins. In our present study, adipose tissues were also incubated in the presence or absence of BSA without tannic acid, so as to rule out any possibility that the inhibitory effect observed was a result of BSA depletion from the incubation buffer due to complex formation between tannic acid and BSA (fig. 3).

Interestingly, gallic acid did not inhibit insulin-stimulated lipogenesis. The gallic moiety by itself is not sufficient to produce the inhibition exerted by tannic acid. This seems to indicate that the integrity of the tannic acid molecule is necessary for its inhibitory effect. The specific binding of insulin to its receptor was found not to be inhibited by tannic acid for concentrations up to 200 μM (fig. 4). Further binding studies have to be undertaken before the mechanism underlying the increase in specific binding at higher tannic acid concentrations can be fully understood. However, the results of the binding assay presented here suffice to provide conclusive evidence that the inhibition of insulin action was not via the inhibition of the binding of insulin to the insulin receptor.

Tannic acid was found to inhibit the in vitro phosphorylation of RR-SRC peptide by insulin receptor tyrosine kinase (fig. 5). A concentration as low as 25 μM was sufficient to inhibit the activity of the insulin receptor tyrosine kinase significantly. The inhibition was partial, with a maximal inhibition of approximately 50% at tannic acid concentrations of 50 μM and above. An active receptor tyrosine kinase is essential for insulin to stimulate glucose and lipid metabolism in rat adipocytes[3,29,38–40]. Interference in the assay by tannin-protein complex formation between tannic acid and RR-SRC peptide was ruled out. Samples in which tannic acid was added prior to the termination of the assay did not differ significantly from the control (data not shown). Chelation of Mg^{2+} and Mn^{2+} by tannic acid could also interfere with the validity of the results. This was excluded by supplementing the assay mixture with additional Mg^{2+} and Mn^{2+} (fig. 6). Interestingly, the increased concentrations of Mg^{2+} and Mn^{2+} enhanced the inhibitory effects of tannic acid. This was in agreement with the study by Martin et al.[41] in which divalent cations such as Mg^{2+} and Ca^{2+} were found to enhance protein-tannin complex formation. This observation provides further evidence for direct interaction between tannic acid and the insulin receptor.

In a similar manner, tannic acid was able to inhibit autophosphorylation of the β-subunit at concentrations as low as 12.5 μM (fig. 7a). Complete inhibition was obtained for concentrations above 25 μM. Autophosphorylation of the β-subunit is necessary for the enhancement of the tyrosine kinase activity[3,4]. Since tannic acid is known to bind to proteins, it would again seem obvious to question whether tannic acid would interfere with the immunoprecipitation of phosphorylated receptors thereby affecting detection. Therefore we ran a parallel assay in which the tannic acid (100 μM) was added after the autophosphorylation had been terminated (fig. 7b). The presence of a 95 kDa band corresponding to the β-subunit of the insulin receptor indicated that the data were acceptable. As mentioned earlier, chelation of Mg^{2+} and Mn^{2+} by tannic acid cannot be overlooked as this would also affect autophosphorylation. The reappearance of 95 kDa bands when higher amounts of insulin receptors were incubated with 50 μM tannic acid indicated that there was sufficient Mg^{2+} and Mn^{2+} present to support autophosphorylation (fig. 8).

From the experimental evidence that we have presented, it can be concluded that tannic acid inhibits insulin-induced lipogenesis in rat adipose tissue by inhibiting the autophosphorylation of the β-subunit of the insulin-

receptor, and to a smaller extent by inhibiting its tyrosine kinase activity, but without inhibiting the binding of insulin. Although an increased amount of BSA was able to mask this inhibitory effect of tannic acid in vitro, this may not occur in vivo. The therapeutic effects of some medicinal plants have been attributed to their gallotannin content[13] despite the readiness of these gallotannins to form complexes with proteins. Therefore further work needs to be done to investigate the inhibitory effect of tannic acid in vivo. These investigations should also include the gastrointestinal absorption of tannins and their concentration in blood as there is no known documentation of these phenomena. Recently, another plant polyphenol (quercetin) was shown to inhibit the insulin receptor tyrosine kinase but not its autophosphorylation[29]. Here we present a compound that could inhibit insulin receptor autophosphorylation without completely inhibiting its tyrosine kinase activity. Our data do not support the current speculation that tannins affect peptide hormone actions by binding to the hormone. Therefore, our novel finding may lead to a new perspective in the understanding of the mode of action of tannins on such hormones.

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